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Chicken Interferon Type I Inhibits Infectious Bronchitis Virus Replication and Associated Respiratory Illness

JIANWU PEI,[1] MARGARET J. SEKELLICK,[2] PHILIP I. MARCUS,[2] IN-SOO CHOI,[1] and ELLEN W. COLLISSON[1]

ABSTRACT

Infectious bronchitis virus (IBV) causes an economically important respiratory disease in poultry worldwide. Previous studies have shown that $CD8^+$ cytotoxic T lymphocytes (CTL) are critical in controlling acute IBV infection, but the role of innate immunity is unknown. This study describes the *in vitro* and *in vivo* anti-IBV activity of natural spleen cell-derived and recombinant chicken interferon type I (rChIFN-α). Both natural and rChIFN-α inhibited replication of the Beaudette strain of IBV in chicken kidney cells (CKC) in a dose-dependent manner, with the antiviral activity of the former accounted for entirely by its content of type I IFN. IFN at 100 U/ml reduced viral replication by 50% as measured by syncytia formation. In addition, the spleen cell-derived supernatants (natural IFN) inhibited tracheal ring ciliostasis mediated by the Gray strain of IBV. Optimal protection against IBV-induced respiratory disease was obtained after intravenous or oral administration of ChIFN given 1 day before virus challenge and each of 5 days thereafter. ChIFN-I protected chicks from clinical illness by delaying the onset of the disease and decreasing the severity of illness, demonstrating its potential as an immune enhancer.

INTRODUCTION

INFECTIOUS BRONCHITIS VIRUS (IBV)-induced respiratory disease is highly contagious in chickens, especially in very young chicks. Respiratory infection with IBV, a prototype of the Coronaviridae family, causes characteristic but not pathognomonic signs, such as sneezing, coughing, tracheal rales, nasal discharge, and labored breathing.[1] In addition to the respiratory tract, some strains of the virus affect the reproductive and urinary systems. In broiler chickens, viral infection can cause decreases in feed consumption and weight gain and, in laying flocks, can result in the decline of egg production and production of thin-shelled, rough, and misshapen eggs.[2] In spite of the wide use of vaccines, outbreaks of the disease continue to occur in vaccinated flocks. Vaccine failures are, in part, caused by antigenic variation of the viral genome, resulting from point mutations and recombination.[3,4]

Innate immunity provides early protection for the host after infection and is essential to the development of adaptive immunity.[5] As a component of innate immunity, type I interferons (IFN), induced by viral infection, can be expressed by many cell types. These cytokines bind to specific receptors on cell surfaces in a species-specific manner before activating the intracellular antiviral pathways that limit the spread of virus before antigen-specific immune responses are established.[6–9] Type I IFN have been used therapeutically to control a range of diseases, including chronic viral hepatitis, cancer, laryngeal and genital papillomas, and multiple sclerosis (MS).[10] Recent studies in mammals also have demonstrated that type I IFN stimulates memory and naive T cell proliferation *in vivo*[9,11–13] and, therefore, might be useful as an immune adjuvant.[11,14,15]

Chicken IFN-I (ChIFN-I) genes have been cloned, sequenced, and expressed as proteins.[16,17] Similar to the mammalian counterparts, ChIFN-I inhibits replication of viruses, including Rous sarcoma virus (RSV),[18] Marek's disease virus (MDV),[19,20] Newcastle disease virus (NDV),[21] avian influenza virus,[22,23] vesicular stomatitis virus (VSV),[24] and Semliki Forest virus (SFV).[24] Daily oral administration of a relatively low dose of recombinant ChIFN-I (rChIFN-α) to 1-day-old chicks was reported to ameliorate Newcastle disease.[21] However, the role of ChIFN-I in protection of chickens from IBV infection by this route, or intravenously (i.v.), has not been established. In the current study, we show that nonstimulated chicken spleen cells produce IFN-I after cultur-

[1]Department of Veterinary Pathobiology, Texas A&M University, College Station, TX 77843-4467.
[2]Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269.

ing *in vitro* for 2 days. The natural IFN-I produced by spleen cells and rChIFN-α inhibited IBV replication in chicken kidney cells (CKC) and tracheal rings. *In vivo* inhibition of respiratory illness associated with IBV infection by ChIFN-I also was examined.

MATERIALS AND METHODS

Cell cultures

Spleen cells were enriched for mononuclear cells by centrifuging on a Ficoll-Hypaque gradient before culturing at 38°C, 5% CO_2 in RPMI 1640 with 10% fetal bovine serum (FBS), 0.1 mM MEM nonessential amino acids, 10^{-5} M β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin.[25] After incubation of these cells for 2 days, the supernatants were collected by centrifuging at 3000 rpm for 10 min. Adherent and nonadherent mononuclear cells were separated by culturing the spleen cell preparations on plastic tissue culture flasks for 4 h.[26] Mitogen-stimulated lymphocytes were treated with 5 μg/ml concanavalin A (ConA) for 2 days.

CKC were prepared from 10-day-old chicks (B19/B19) as described previously[27] and cultured in DMEM with 10% FBS, 1.4% sodium pyruvate, 0.1 mM MEM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Chicken embryo cells (CEC) were prepared and cultured as described.[28] The chicken macrophage cell line HD-11 was cultured in RPMI 1640 with 10% FBS. Tracheal rings were made from 19-day-old embryos and cultured in a 48-well plate with one ring per well in DMEM.[29] Vero cells were cultured with DMEM and 10% FBS.

Virus

The tissue culture-adapted IBV Beaudette strain was propagated, and the titer was determined in CKC. VSV was prepared as described previously.[28] The IBV Gray strain was propagated following inoculation of the allantoic sac of 11-day-old chicken embryos.[30] Allantoic fluid, harvested 36 h postinfection (p.i.) with a titer of $10^{8.4}$ EID_{50}/ml, was used as virus stock.

IFN assays

The ChIFN-I titers in supernatants were determined with the VSV plaque reduction assay in CEC.[28] Identification of ChIFN-I was based on its stability at pH 2 and neutralization by specific monoclonal antibody (mAb) (Masayoshii Kohase, NIID, Tokyo, Japan). The rChIFN-α,[31] with a titer of 1.8×10^6 U/ml (VSV-PR_{50} assay), was produced from transfected COS cells.[16]

IFN-γ in the supernatants was determined using Griess reagent (Sigma, St. Louis, MO) to quantify the production of nitric oxide (NO) from HD-11 cells.[26] Briefly, 10^5 HD-11 cells in 50 μl RPMI were seeded in a 96-well plate, and an equal volume of supernatant was added. The plate was incubated at 38°C, 5% CO_2 for 24 h before 50 μl HD-11 cell supernatant was collected and added to 50 μl Griess reagent. The absorbance was detected at 470 nm, and the NO concentration was determined using a standard sodium nitrite solution.

rChIFN-γ (type II) purified after expression in *Escherichia coli* (M15) was used for positive IFN-γ controls. rChIFN-γ cDNA was generated by RT-PCR.[32] The product was cloned into pQE30 expression plasmid (Qiagen, Chatsworth, CA), and ChIFN-γ was purified with Ni-NTA resin using the manufacturer's suggested procedures (QIAexpressionist Protocol) (Qiagen). The recombinant product was used at a concentration of 30 ng/ml.

Anti-IBV activity in infected CKC

CKC (3×10^4 per well) cultured in a 96-well plate were inoculated with the IBV Beaudette strain (50 $TCID_{50}$/well). After 2 h incubation at 38°C, 5% CO_2, the virus was replaced with complete DMEM (described for CKC) containing varying doses of rChIFN-α or spleen cell supernatant. Viral replication was determined by counting the number of syncytia in each well after an overnight incubation.[33] The viability of the CKC was determined by the trypan blue exclusion assay.

Anti-IBV activity in infected tracheal rings

IBV infection will decrease the movement of cilia in tracheal rings cultured *in vitro* in DMEM with 10% FBS.[2,29] Eight tracheal rings collected from 19-day-old embryos were infected with the IBV Gray strain at 100 CD_{50} (median ciliastatic dose) per ring.[29] Four rings without virus inoculation served as negative controls. The virus in four of the eight infected rings was replaced with 50% spleen cell supernatant in DMEM 2 h p.i. The tracheal rings were examined daily for ciliastasis and scored as: −, no movement of cilia; +, cilia movement observed but obviously slower than controls; ++, movement similar to uninfected control.

Anti-IBV activity in infected chicks

B19/B19 chicks (Dr. W.E. Briles, Department of Biological Sciences, Northern Illinois University) and SPF chicks (Charles River SPAFAS, North Franklin, CT) were housed in a specific pathogen-free environment at the Laboratory Animal and Resources and Research Facility (Texas A&M University, College Station, TX). Six to eight-day-old chicks were inoculated either

TABLE 1. CHIFN-I TITER IN SUPERNATANTS AFTER 2 DAYS *IN VITRO* INCUBATION

Cells (10^7/ml)	*IFN-I titer (U/ml)*	*Neutralized by mAb (%)*
Nonadherent cells	50	99.98
Adherent cells	250	90.00
Spleen cells	2,120	99.94
ChIFN-I standard	24,230	99.97

TABLE 2. IFN-γ WAS PRODUCED BY SPLEEN CELLS AND NONADHERENT CELLS AFTER CONA STIMULATION[a]

		Nitric oxide (μM)[b]	
Source supernatant	*ConA stimulation*	*Before pH 2*	*After pH 2[c]*
Spleen cells	−	0.032 ± 0.061[d]	N/A[e]
Spleen cells	+	3.972 ± 0.192	0.035 ± 0.066
Nonadherent cells	−	0.018 ± 0.039	N/A
Nonadherent cells	+	3.972 ± 0.012	0.009 ± 0.029
IFN-γ control[f]	N/A	9.388 ± 0.258	0.115 ± 0.039

[a]As inferred from NO production and acid lability.
[b]Nitric oxide levels (μM) produced by HD-11 cells after stimulation by the supernatants or rIFN-γ.
[c]The supernatants or IFN-γ control were treated overnight at pH 2, and the pH was adjusted back to 7.0 with NaOH before stimulating HD-11 cells.
[d]Mean ± SD.
[e]N/A, not available.
[f]Control IFN-γ was recombinant protein (30 ng/ml) purified from *E. coli*.

i.v. or orally with a needlelesssyringe at varying doses of rChIFN-α in RPMI or spleen cell supernatant. Chicks inoculated with medium served as controls. All the chicks were challenged with IBV Gray strain (10^6 EID_{50} per chick) by the nasal-ocular route. The chicks were observed daily for clinical signs of illness after challenge and scored as: 0, no clinical illness; +, sneezing or coughing; ++, plus rales; +++, plus dyspnea. The percentage of maximum possible illness for each group was calculated as:

$$(A/B) \times 100$$

where *A* was the clinical score (i.e., pluses) observed for all chicks in a group, and *B* was the maximum possible clinical score (i.e., pluses) for the total number of chicks in a group. Body weights for each chick were recorded when killed at 7 days p.i.

Statistical analysis

Student's *t*-test was used to compare the CKC viability, and analysis of variance (ANOVA) was used to analyze the clinical score and body weight. $p < 0.05$ was considered as significant.

RESULTS

Chicken spleen cells without stimulation produced type I but not type II ChIFN after 2 days in vitro *incubation*

The capacity for chicken spleen cells to secrete antiviral cytokines was examined. IFN activity was evaluated with the standardized VSV plaque reduction assay. Two days after culturing *in*

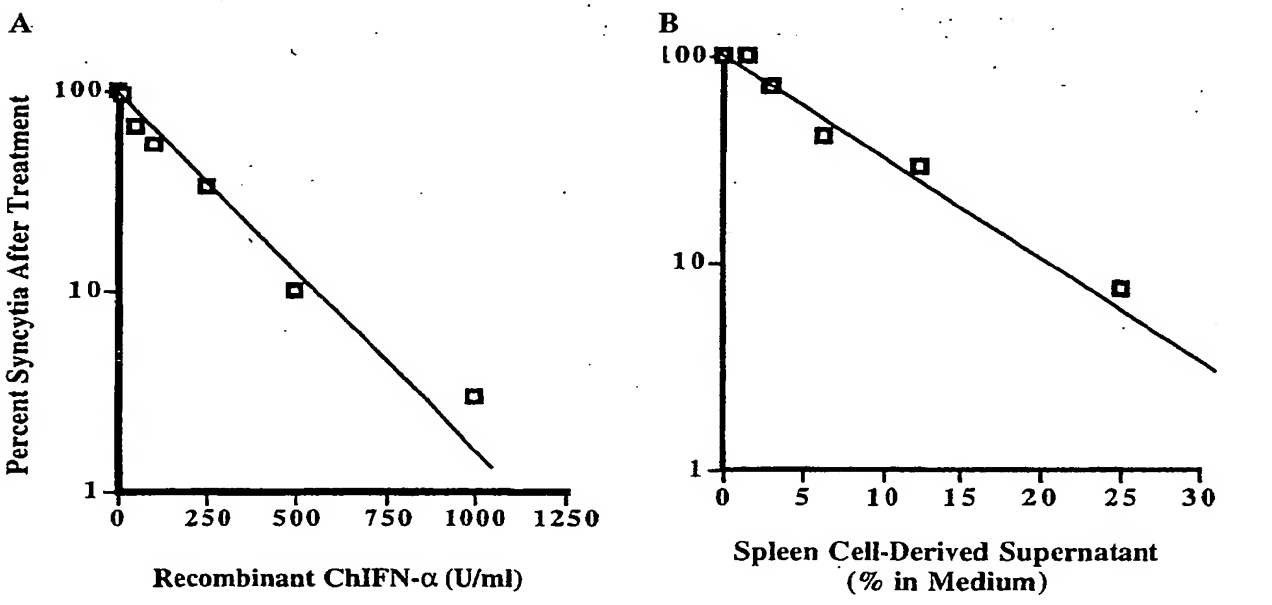

FIG. 1. rChIFN-α and supernatant from chicken spleen cells inhibited IBV replication in a dose-dependent manner. Varying dilutions were added to IBV-infected CKC. The number of syncytia correlated with IBV infection when counted after overnight incubation. (A) rChIFN-α inhibition of IBV replication. (B) Inhibition of IBV following treatment with natural ChIFN-I from spleen cells cultured *in vitro* for 2 days.

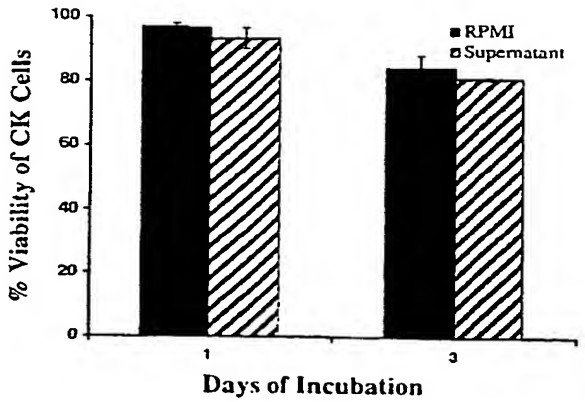

FIG. 2. Viability of CKC incubated with 50% supernatant did not decrease significantly when compared with that of cells grown in RPMI without supernatant ($p > 0.1$).

vitro in the absence of mitogen, supernatants were collected from unfractionated chicken spleen cells and from adherent and nonadherent fractions. IFN-like activity was detected and titered by a standard VSV plaque reduction assay (Table 1). The titer of natural ChIFN-I in the supernatant of 10^7 unfractionated spleen cells was 2120 U/ml. In contrast, 250 U/ml and 50 U/ml were detected in the supernatants of an equivalent number of adherent and nonadherent cell fractions, respectively. The results suggest that both adherent and nonadherent cells are necessary for optimal production of antiviral activity. The antiviral activity in the supernatants was shown to be caused by a type I ChIFN because it was acid stable and neutralized (≥90%) by specific mAb (Table 1).

Spleen cells produced IFN-γ after ConA stimulation

Type I IFN, in contrast to IFN-γ, induces little or no NO in macrophages.[24] IFN-γ production from spleen cells or the nonadherent cells was evaluated by determining the production of NO by stimulated HD-11 cells. Both unfractionated spleen cells and nonadherent cells secreted IFN-γ activity following ConA stimulation (Table 2). Unlike IFN-I production, IFN-γ could not be detected in the supernatants of spleen cells or nonadherent cells in the absence of ConA stimulation (Table 2). The IFN-γ activity in the supernatants was confirmed by its capacity to induce NO and its acid lability (Table 2).[26]

ChIFN-I inhibited IBV replication in vitro

To examine further the *in vitro* anti-IBV activity of recombinant and natural (spleen cell-derived) ChIFN-I, CKC infected with the IBV Beaudette strain were treated with natural (spleen cell supernatant) or rChIFN-I. As showed in Figure 1A, rChIFN-I inhibition of IBV replication in CKC was dose dependent. Natural ChIFN-I from chicken spleen cell supernatants similarly inhibited IBV replication in a concentration-dependent manner (Fig. 1B). Natural and recombinant ChIFN-I had similar anti-IBV activity when equated with their respective contents of IFN. The spleen cell-derived IFN was noncytotoxic based on CK cell viability after a 72-h exposure (Fig. 2). Because type I ChIFN is known to be species specific, the potential anti-IBV activity of the spleen cell supernatant was examined in Vero cells. Supernatants collected from chicken spleen cells did not inhibit IBV replication, as measured by syncytia formation in Vero cells (data not shown).

Inhibition of IBV replication in tracheal rings

IBV infectivity can be detected in infected tracheal.[2] Cilia in normal, uninfected tracheal rings continue to move for >7 days in tissue culture. However, cilia will stop moving after 3–4 days p.i. with IBV. To determine the effect of ChIFN-I on the ciliastasis mediated by IBV, eight trachea rings were infected with the Gray strain. Four of them were treated with spleen cell supernatant. Four days after infection, all four infected control rings showed ciliastasis, whereas cilia in three of the four spleen cell supernatant-treated rings were moving similarly to the uninfected controls (Table 3).

ChIFN-I partially protected chicks from IBV infection

To examine the anti-IBV activity of ChIFN-I *in vivo*, 6-day-old B19/B19 chicks were first inoculated i.v. with 0.5 ml spleen cell supernatant (1060 U ChIFN-I) or 0.5 ml rChIFN-I (500 U) 30 min before challenging with the IBV Gray strain. Three days after viral infection, chicks were similarly inoculated with the same doses of natural or rChIFN-I. Clinical illness was scored, and the percentage of maximum possible illness for each group was calculated at various times after infection. Both the natural ChIFN-I from spleen cells and the rChIFN-I inhibited IBV-associated respiratory illness. As shown in Figure 3, observed mean respiratory illness of chicks receiving ChIFN-I was <30% of maximum possible illness. Although a reduction in respiratory illness was observed, no differences were detected in the body weights of the chicks from any of the experimental groups, infected or not infected, or treated or not treated (data not shown).

The *in vivo* effects of ChIFN-I on IBV-induced respiratory illness were evaluated. To examine the anti-IBV activity of ChIFN-I given orally, fifty 8-day-old SPAFAS chicks were divided into five groups. One-tenth milliliter of spleen cell supernatant (about 200 U of ChIFN-I) or varying doses of

TABLE 3. IBV GRAY STRAIN REPLICATION IN TRACHEAL RINGS WAS INHIBITED BY SPLEEN CELL SUPERNATANT

Group	*Ciliastasis scores at 4 days p.i.*[a]				*Viral replication (%)*
Supernatant	++	−	++	++	25
Infected control	−	−	−	−	100
Uninfected control	++	++	++	++	0

[a]Ciliastasis scores: −, cilia stop moving; ++, moving as uninfected control.

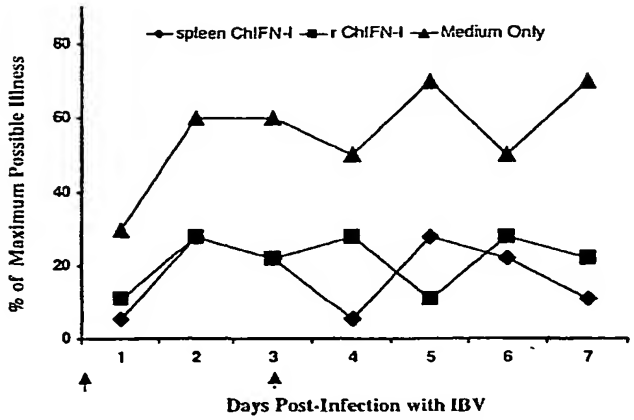

FIG. 3. Inhibition of IBV-associated clinical illness of chicks receiving two doses of natural and recombinant ChIFN-α i.v. One dose was given 30 min before infecting with the IBV Gray strain, and the other dose was given at 3 days p.i. (arrows). The percentage of maximum possible illness is shown for each group at the indicated times. Statistical analysis indicated that the chicks receiving natural ChIFN-I (1060 U) were significantly protected at 3, 4, and 7 days p.i., whereas the chicks receiving rChIFN-α (500 U) were protected at 3 and 5 days p.i.

rChIFN-I (10^2, 10^3, and 10^4 U) in RPMI 1640 were administered orally 1 day prior to IBV Gray strain infection and each of 5 days thereafter. The chicks receiving RPMI 1640 served as controls. Protection could be observed in infected chicks given either source of IFN-I. As shown in Figure 4, the spleen cell supernatant consistently provided the greatest protection, with a significant difference from the untreated chicks 2–6 days p.i. ($p < 0.05$). Although there were no significant differences in observed protection among chicks receiving the three doses of rChIFN-I, variation increased as the dose decreased. Chicks receiving 10^4 U rChIFN-I were significantly protected ($p < 0.05$) 2, 3, 4, and 5 days p.i. Chicks receiving 10^3 U rChIFN-I were protected 2, 3, and 5 days p.i., whereas 10^2 U rChIFN-I provided protection only on days 2 and 5 p.i. Significant differences in the mean body weight were not observed among the groups, although there was a broader distribution of weight in the control group given RPMI only (Fig. 5).

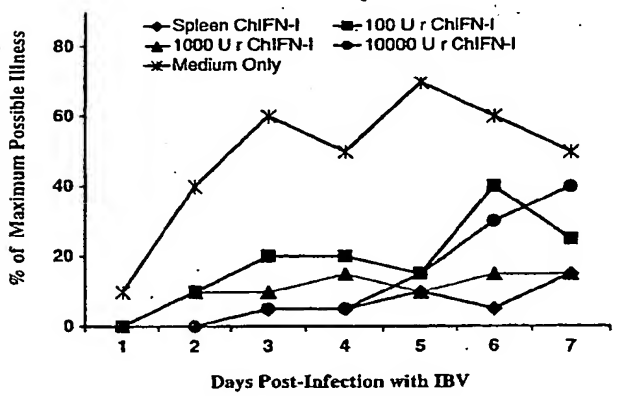

FIG. 4. Inhibition of clinical respiratory illness in the chicks receiving six doses of natural spleen cell-derived and recombinant ChIFN-α. One dose was given orally 1 day before IBV Gray strain infection and daily thereafter for 5 days p.i. Percentage of maximum possible illness is shown for each group at the indicated times. The spleen cell supernatant (200 U) consistently provided the greatest protection, with a significant difference from the untreated chicks 2–6 days p.i. ($p < 0.05$). Chicks receiving 10^4 U rChIFN-I were significantly protected ($p < 0.05$) at 2, 3, 4, and 5 days p.i. Chicks receiving 10^3 U rChIFN-I were protected at 2, 3, and 5 days p.i., and 10^2 U rChIFN-I provided protection only at day 2 and 5 p.i.

DISCUSSION

Type I IFN are produced in response to viral infection by many types of cells, and type II IFN (IFN-γ) is produced by natural killer (NK) and T cells following exposure to antigen-presenting cells (APC).[6] In the current study, nonstimulated chicken spleen cells were found to produce a type I IFN that was neutralized by mAb specific for rChIFN-α. Optimal induction of IFN was observed only in the presence of both adherent and nonadherent cells, perhaps accounting for the absence of either type of IFN from nonstimulated chicken spleen cells, as reported by Lowenthal et al.[26] In agreement with others,[26,34] we found that ChIFN-γ, as defined by its sensitivity to pH 2 and induction of NO in HD-11 macrophages, was generated only in spleen cells stimulated by ConA or APC (data not shown).

The supernatant medium from nonstimulated spleen cells reduced the replication of IBV, as assessed by a reduction of syncytia in CKC and cilia motion in tracheal rings, suggesting the possible involvement of IFN. IBV has been shown to induce IFN in cultured cells (CKC and CEC), embryonated eggs, tracheal organ culture, and infected chickens,[35–37] suggesting that IFN induction may be an important innate immune response to this coronavirus. However, studies describing IBV sensitivity

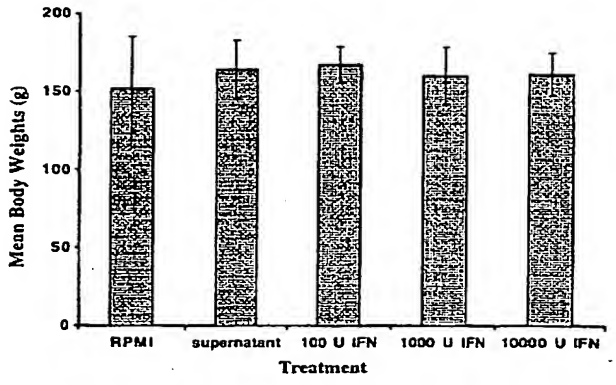

FIG. 5. Body weights of chicks receiving six daily oral doses of spleen cell supernatant or rChIFN-I were not affected. The body weights were recorded at 7 days p.i. The bars represent mean values in the indicated group. The differences among the groups were not significant ($p > 0.05$).

to ChIFN have been inconsistent. Otsuki et al.[36] reported that all 10 strains of IBV tested were sensitive to IFN action in CKC. In contrast, Holmes and Darbyshire[35] found that none of six strains of IBV investigated were sensitive to ChIFN as tested in cultures of tracheal rings.

The use of rChIFN-α[16,17] demonstrated its antiviral action against IBV, both *in vitro* and *in vivo*. The chicken spleen cell-derived anti-IBV activity could be accounted for entirely by its content of natural ChIFN. The action of this natural IFN and rChIFN-α were equivalent when compared on the basis of their activity against VSV in a standard PR_{50} assay. Both sources of IFN generated survival curves for IBV on CKC that revealed PR_{50} of 100 U/ml IFN, meaning that IBV was 100 times less sensitive to the action of ChIFN-α than was the highly sensitive VSV. Nonetheless, IBV was significantly more sensitive to IFN-α than other avian viruses, such as reovirus and NDV.[38] Furthermore, the exponential nature of the IBV survival curve represents over 90% of the virus population, suggesting that IFN-resistant subpopulations sometimes observed in quasi-species of RNA viruses may not be of concern with this coronavirus.[23,38] ChIFN-I can be added as long as 8 h p.i. and still be effective (data not shown). This suggests that the IFN may act within the first replication cycle of the virus or that newly released virus is prevented from replicating in IFN-treated cells it encounters on release from the initially infected cell. Because the mechanism of inhibition of IBV by IFN action is not known, it will be of interest to determine whether ChIFN-α and ChIFN-γ act additively[39] or synergistically[24] against this virus in chickens and if the sequential treatment with IFN-α and dsRNA further enhances the antiviral effect of IFN, as has been demonstrated *in vitro* against reovirus and NDV, two avian viruses that are refractory to IFN action.[38]

Type I IFN have been given orally in relatively low doses to ameliorate or prevent a number of infectious and immune-based diseases in a variety of mammals.[40,41] Low doses of rChIFN-α have been administered for 2 weeks in the drinking water of chicks beginning at 1 day of age, and this was found to ameliorate Newcastle disease and reduce viral replication, with higher doses being more effective than lower doses.[21] Oral administration of rChIFN-α also reduced MDV R2/23 replication *in vivo*.[20] We found that 200 U natural or 10^4 U rChIFN-α given orally protected chicks from severe IBV-induced clinical illness. One hundred units of rChIFN-α was marginally less effective because with greater daily variation, significant differences from the controls were observed only on days 2 and 5 p.i., whereas chicks receiving 10,000 U rChIFN-α were protected from days 2 to 5 p.i. The optimal amount of IFN needed for viral protection may vary with the animal model. In mice, doses of 10 and 1 U provided protection against cytomegalovirus infection, whereas 100 U and greater did not.[42]

Under typical congested poultry housing conditions, many pathogens and environmental stresses are of serious economic concern. In addition to the use of aggressive vaccine strategies, innate immunity and reduced environmental stresses could optimize the immune protection. Because rChIFN-α can be produced economically in large amounts, is pH and heat stable, has a long $t_{1/2}$ in water, and is nontoxic at doses effective orally,[21] it reasonably could be exploited as a prophylactic or therapeutic treatment of avian viral diseases.

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Address reprint requests to:
Dr. Ellen W. Collisson
Department of Veterinary Pathobiology
Texas A&M University
College Station, TX 77843-4467

Tel: (979) 845-4122
Fax: (979) 862-1088
E-mail: ecollisson@cvm.tamu.edu

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